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Standardization of the Human Cytomegalovirus Antigenemia Assay by Means of In Vitro-Generated pp65-Positive Peripheral Blood Polymorphonuclear Leukocytes

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We generated in vitro human cytomegalovirus (HCMV) pp65-positive polymorphonuclear leukocytes (PMN) resembling those detected in vivo, following cocultivation of PMN from healthy donors and wild-type HCMV-infected endothelial cells or fibroblasts. After purification, PMN are suitable for preparation of cytospots which can be used for the antigenemia assay. Cytospin preparations containing a predetermined number of in vitro-generated pp65-positive PMN were used to test some of the major parameters involved in performing the antigenemia assay. The results showed or confirmed that (i) formalin fixation followed by permeabilization is the best fixation procedure developed to date, (ii) the test performance levels provided by different pools of pp65-specific monoclonal antibodies may be significantly different, and (iii) long-term storage (for an unlimited time) is best achieved by keeping fixed slides at −80°C, whereas short-term storage (for up to 1 month) is best achieved by keeping unfixed slides at room temperature. This finding signifies that slides can be shipped all over the world at room temperature. In conclusion, the newly developed procedure for in vitro generation of pp65-positive PMN will provide the basis for standardization of the HCMV antigenemia assay and development of quality control programs.

Since its introduction in 1988 (13, 14), the procedure for antigenemia assay for detection of pp65 of human cytomegalovirus (HCMV) in polymorphonuclear leukocytes (PMN) has undergone multiple modifications and improvements of cell fixation, immunostaining procedure, and use of pooled monoclonal antibodies (3). However, standardization has not been achieved thus far, and results obtained in different laboratories are difficult to compare. The major obstacle to the standardization of the antigenemia assay has been the lack of biological material (pp65-positive PMN) available in unlimited quantities and standardized with a predetermined ratio of pp65-positive PMN/pp65-negative PMN. Thus far, the only positive control introduced into a commercially available kit for determination of pp65-antigenemia (5) consisted of cytospin preparations containing insect cells expressing HCMV pp65 mixed with PMN from healthy volunteers (12).

In the present report, we describe the use of the recently developed procedure for generating in vitro pp65-positive PMN (9) for the optimization and standardization of the HCMV antigenemia assay. The availability of a proper positive control can lead to development of standardization protocols and quality control programs in the near future.

MATERIALS AND METHODS

Isolation of PMN. Concentrated preparations of human PMN from either HCMV-seropositive or HCMV-seronegative healthy donors were obtained as follows. One milliliter of 6% dextran solution (molecular weight, 70,000) in saline was added to 5.0 ml of heparinized blood. Following incubation at 37°C for 30 min, the supernatant was dispensed onto Ficoll-Hypaque and centrifuged at $600 \times g$ for 35 min. Contaminating erythrocytes were removed by hypotonic lysis (1.0 ml of 0.8% NH_4Cl for 2 min). Both the purity and the viability of harvested PMN were consistently found to be >95%, as assessed by May-Grünwald staining and trypan blue exclusion, respectively (9).

Cocultivation of PMN and cell cultures infected with different HCMV strains. PMN were then cocultivated with HCMV-infected human umbilical vein endothelial cells (HUVEC) or human embryonic lung fibroblasts (HELF). HUVEC were obtained by trypsin treatment of umbilical cord veins and used at passages 5 to 10, while HELF were derived from a cell strain developed in our laboratory and used at passages 20 to 30. HCMV strains, either laboratory-adapted (AD169, Davis, and Towne from the American Type Culture Collection, Rockville, Md.) or wild-type (HCMV isolates from blood and different body sites) strains, were routinely propagated in HELF cultures. In addition, one wild-type strain (VR6110) was adapted to growth on HUVEC. Cocultivation of PMN and HCMV-infected HELF or HUVEC (ratio, 2:1 to 5:1) was continued for 24 h at 37°C.

pp65-positive PMN detection and purification. Following cocultivation, pp65-positive HELF or HUVEC were easily distinguishable from pp65-positive PMN by their size and predominantly cytoplasmic staining in contrast to the restricted PMN nuclear staining (3). However, in order to separate PMN from infected cells, cell mixtures (5×10^5 to 1×10^6 cells) in 250 μl of RPMI 1640 (Flow Laboratories, Irvine, Scotland) plus 1% fetal calf serum were placed in the upper compartment of a cell culture insert (5-μm-pore-size, 6.5-mm-diameter Transwell filters; Costar, Cambridge, Mass.) and incubated for 3 h at 37°C in a 5% CO_2 humidified atmosphere, while the bottom compartment contained 10^{-8} M formyl-methionyl-leucyl-phenylalanine (Sigma Chemical Co., St. Louis, Mo.) in 500 μl of RPMI 1640 plus 1% fetal calf serum (9, 11). After incubation, chambers were shaken to dislodge PMN from the lower surface of the inserts. PMN were then collected, washed, counted, and used for cytospins preparation. In preliminary experiments, PMN collected after migration were further purified by fluorescence-activated cell sorting (FACS) following PMN staining with CD66b (Immunotech, Milan, Italy). The number of pp65-positive PMN was determined on aliquots of migrated PMN before and after FACS purification.

Standardization of the antigenemia assay with in vitro-generated pp65-positive PMN. Using in vitro-generated pp65-positive PMN, we addressed the problem of the standardization of the antigenemia assay in its three basic parameters: (i) fixation, (ii) immunostaining technique, and (iii) pool of monoclonal antibodies. The two types of fixation most commonly used, methanol-acetone (13) and formalin plus Nonidet P-40 (NP-40) (3), were compared. In addition, the two most commonly used immunostaining techniques, the immunoperoxidase (either the indirect method [immunoperoxidase assay; IPA] or the avidin-biotin complex method [ABC]) and the immunofluorescence assay (IFA) method (3), were compared. Finally, once the most efficient fixation and immunostaining methods were selected, the most commonly used pools of pp65-specific monoclonal antibodies were compared. These were (i) our pool (referred to as PV pool) including the three monoclonal antibodies 1C3, 2A6, and 4C1 (3), (ii) Clonab CMV (Biotest AG, Dreieich, Germany [4]), (iii) CINApool (Argene Biosoft, Varilhes, France), and (iv) Chemicon (Chemicon International Inc., Temecula, Calif.). Furthermore, the optimal storage temperature for either fixed or unfixed cytospots was investigated in view of performing quality control programs requiring shipment of known positive controls. In each set of experiments the following parameters were determined: (i) the number of pp65-positive PMN per

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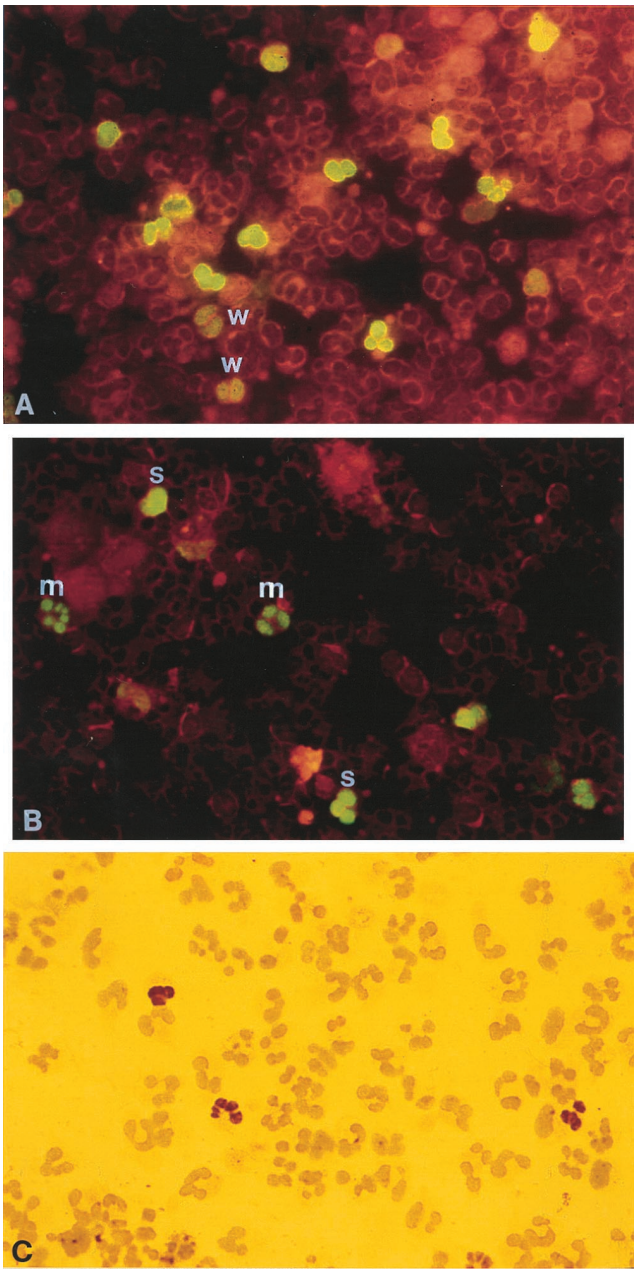

FIG. 1. Indirect immunofluorescence (A and B) and immunoperoxidase (C) staining of cytospin preparations of PMN containing pp65-positive cells obtained from an AIDS patient with disseminated HCMV infection (A) or generated in vitro (B and C). Degree of staining is indicated as weak (w), moderate (m), or strong (s) according to intensity of fluorescence. Magnification, ×910.

2×10^5 PMN used for preparation of each cytospot, and (ii) the degree (intensity) of staining, which was determined by classifying positive cells into the three staining categories of weak (1+), moderate (2+ or 3+), and strong (4+).

Statistical analysis. Comparison between means was performed with the Student *t* test, whereas the difference in distribution of staining categories was analyzed by the Pearson chi-square test.

RESULTS

In vitro generation of pp65-positive PMN. Initial attempts at generating pp65-positive PMN following coculture with HELF infected with the reference HCMV strain AD169 were consistently unsuccessful. Similarly negative results were obtained when other laboratory-adapted strains, such as Davis or Towne, were used. pp65-positive PMN were generated when PMN were cocultured with HUVEC infected with a clinical HCMV isolate from blood (VR6110) (Fig. 1). Similar results were also achieved with HELF infected with different isolates from different body sites of both immunocompromised subjects and immunocompetent subjects. No pp65-positive PMN were observed when contact of PMN with infected cells was prevented by using Transwell inserts (0.4-μm-pore-size membranes) or when contact was restricted to infectious cell-free wild-type virus.

PMN purification and testing. Once generated, pp65-positive PMN had to be separated from contaminating HCMV-infected cells. Since the level of purification achieved after Transwell insert migration was not substantially increased by the additional FACS purification, Transwell migration was subsequently used as a routine purification step. Then, serial concentrations of pp65-positive PMN were obtained in cytospots set up following preparation of serial mixtures, at a different proportion, of a known pp65-positive migrated PMN suspension and a PMN suspension from healthy donors. Results of testing of replicate cytospin preparations containing a different number of pp65-positive PMN are reported in Table 1, where the coefficient of variability of the mean is consistently below 10% when levels of antigenemia are >50, while it becomes progressively higher with antigenemia levels approaching 1.

Fixation. The better performance of the formalin plus NP-40 fixation over the methanol-acetone method was confirmed in terms of both number of pp65-positive PMN and staining intensity (data not shown). In addition, some experiments were carried out to verify whether NP-40 permeabilization of PMN previously fixed with formalin was a critical step. It was found that, although the fluorescent staining was more evenly distributed over the entire nucleus of positive cells in permeabilized PMN preparations, both the number of positive cells and the degree of staining were not significantly different in the two groups of cytospin preparations (data not shown).

Immunostaining technique. No significant differences were found among IFA, IPA, and ABC (Table 2). In addition, although the distributions of different PMN staining groups were not significantly different among the three tested techniques, the absolute number of PMN belonging to the weak IFA staining group was greater than the relevant IPA and ABC group values, whereas the number of strongly stained cells was slightly lower for the IFA technique.

Pool of monoclonal antibodies. The results of the comparison between the sensitivity of our pool (referred to as PV pool)

TABLE 1. Variability in the frequency of pp65-positive PMN in replicates of cytospin preparations containing serial proportions of positive and negative PMN obtained by using the PV pool

Cytospin preparation	No. of pp65-positive PMN per 2×10^5 PMN for replicate:					Mean ± SD	Coefficient of variation
	1	2	3	4	5		
1	510	490	520	520	480	504.0 ± 18.16	0.036
2	400	410	408	415	406	407.8 ± 5.49	0.013
3	177	170	175	185	170	175.4 ± 6.18	0.035
4	100	102	96	105	106	101.8 ± 4.02	0.039
5	69	55	68	64	58	62.8 ± 6.14	0.097
6	30	31	29	40	31	32.2 ± 4.43	0.137
7	19	17	12	18	8	14.8 ± 4.65	0.314
8	4	2	5	4	4	3.8 ± 1.09	0.288

TABLE 2. Sensitivities of different immunostaining techniques with formalin plus NP-40 as fixative (prior to storage at −80°C) and the PV pool

Staining technique (No. of replicates)	Median no. of pp65-positive PMN (range)	*P* value[a]		Median no. (%) of pp65-positive PMN with degree of staining			*P* value[b]	
				Weak	Moderate	Strong		
IFA (10)	205.5 (160–240)			51.5 (25)	130.5 (64)	23.5 (11)		
IPA (10)	200 (145–230)	NS[c]	NS	33 (16)	143 (72)	24 (12)	NS	NS
ABC (10)	203 (148–245)	NS		36 (18)	139.5 (69)	27.5 (13)	NS	

[a] *t* test.
[b] Pearson chi-square test.
[c] NS, not significant.

containing three monoclonal antibodies and the sensitivities of three commercially available pools, each containing two monoclonal antibodies, are reported in Table 3. The PV pool resulted in significantly higher number of positive PMN than the Argene ($P = 0.0041$), the Biotest ($P < 0.00001$), and the Chemicon ($P = 0.0008$) pools. In addition, the Argene pool gave significantly better results than the Biotest pool ($P = 0.0047$), whereas no statistically significant difference was found between the Argene and Chemicon or between the Biotest and Chemicon pools. As for distribution of staining intensity, the PV pool gave 25% weakly, 64% moderately, and 11% strongly stained cells, whereas the Argene, Chemicon, and Biotest pools detected a higher percentage of weakly stained cells (38 to 43%) and lower proportions of moderately (52 to 54%) and strongly (3 to 10%) stained cells. However, these differences were not statistically significant.

Storage temperature. The optimal storage temperature was a major issue addressed by this study. Initially, fixed and unfixed slides were tested in parallel following a 7-day storage period at room temperature or at −80°C (Table 4). It was found that fixation prior to storage at −80°C gave significantly better results than fixation after storage at −80°C and prior to staining ($P = 0.0186$). In contrast, fixation prior to staining and following storage at room temperature was by far better than fixation done just after cytospin preparation ($P = 0.0009$). In other words, the best condition was a 7-day storage of unfixed slides at room temperature followed by storage at −80°C of fixed slides. No significant difference was found between the two conditions. Significantly lower sensitivities were found for either unfixed slides at −80°C or fixed slides at room temperature. In terms of staining intensity, there was a significant difference among the different experimental conditions tested ($P = 0.0016$): a significant difference was found between unfixed and fixed slides stored at room temperature ($P = 0.012$), between unfixed slides stored at room temperature and at −80°C ($P = 0.004$), and between fixed and unfixed slides stored at −80°C ($P = 0.005$). In general, a 7-day storage of unfixed slides at room temperature was associated with a relatively smaller number of weakly stained cells and a relatively greater number of moderately stained PMN (Table 4) compared to the other experimental conditions.

Since short-term storage at room temperature of unfixed slides provided the best results, this condition was tested for longer time with slides fixed with formalin plus NP-40 at the time of immunostaining with the PV pool of monoclonal antibodies and the IFA technique (Table 5). It was found that the number of positive cells remained stable, i.e., not significantly different from the baseline value, for up to 30 days of storage. Then, it decreased progressively, dropping to zero positive cells after 75 days. The relative proportion of weakly stained PMN progressively increased after 30 to 40 days of storage at room temperature, while in the meantime the relative proportion of PMN stained at a moderate degree decreased.

DISCUSSION

Some major conclusions can be drawn from this study: (i) a proper positive control for the antigenemia assay is now available; (ii) it is now feasible to prepare large batches of cytospots containing a predetermined number of pp65-positive PMN; (iii) all parameters involved in the performance of the assay can be carefully controlled, thus allowing standardization of the test system for the first time; (iv) quality control programs involving laboratories in different countries and on different continents can be developed; and (v) unfixed slides can be shipped at room temperature from a single reference center

TABLE 3. Sensitivities of different pools of HCMV pp65-specific monoclonal antibodies with formalin plus NP-40 as fixative (prior to storage at −80°C) and IFA as immunostaining technique

Monoclonal antibody pool (no. of monoclonal antibodies)[a]	Median no. of pp65-positive PMN (range)	*P* value[b]			Median no. (%) of pp65-positive PMN with degree of staining			*P* value[c]		
					Weak	Moderate	Strong			
PV pool (3)	205.5 (160–240)				51.5 (25)	130.5 (64)	23.5 (11)			
Argene (2)	169 (140–210)	0.0041	0.0047	NS[d]	64 (38)	87.5 (52)	17.5 (10)	0.022	0.046	NS
Biotest (2)	138 (117–153)	<0.00001	NS		58.5 (43)	75 (54)	4.5 (3)	0.0003	NS	
Chemicon (2)	152.5 (110–179)	0.0008			57.5 (38)	83 (54)	12 (8)	0.034		

[a] For each pool, data for 10 replicates are shown.
[b] *t* test.
[c] Pearson chi-square.
[d] NS, not significant.

TABLE 4. Effect of temperature during storage for a 7-day period on fixed and unfixed PMN slides with IFA and the PV pool

Storage temperature	Fixation upon slide preparation	No. of replicates	Median no. of pp65-positive PMN (range)	*P* value[b]			Median no. (%) of pp65-positive PMN with degree of staining			*P* value[c]		
							Weak	Moderate	Strong			
RT[a]	None	5	178.0 (160–196)				30 (17)	133 (75)	15 (8)			
	Formalin + NP-40	5	109 (86–117)	0.0009	0.0003	0.029	34 (31)	70 (64)	5 (5)	0.012	NS[d]	NS
−80°C	None	5	129 (114–144)	0.004	0.0186		38 (29)	87.5 (68)	3.5 (3)	0.004	0.005	
	Formalin + NP-40	6	157.5 (134–192)	NS			38.5 (24)	99 (63)	20 (13)	NS		

[a] RT, room temperature.
[b] *t* test.
[c] Pearson chi-square ($P = 0.0016$, overall comparison of three degrees of staining at different temperatures).
[d] NS, not significant.

for short-term staining or for long-term storage at −80°C following fixation upon delivery.

Using in vitro-generated pp65-positive PMN, we have confirmed that fixation with formalin provides better results than fixation methods involving acetone, as previously reported by our group as well as by others (1, 3, 8). The use of the formalin fixation procedure without the subsequent permeabilization step has recently been recommended because of its simplicity (8). On the basis of this study, we believe that, although the suppression of the permeabilization step does not cause a significant decrease in terms of absolute number of positive cells, a more even distribution of specific staining is obtained in permeabilized cells, while the time required for test performance is only slightly longer (5 min).

As for the immunostaining technique, we previously reported significantly better results given by the IFA compared to the IPA or ABC procedure (3). We attributed this difference to the procedure incorporating use of methanol-H_2O_2 for removal of endogenous peroxidase activity. However, when 3-amino-9-ethylcarbazole in acetate buffer was used for detection of enzymatic activity (13, 15), no significant background staining was encountered and no difference in terms of positive cells was noted. In fact, no differences among IFA, IPA, and ABC were found in this study.

All three commercially available pools of pp65-specific monoclonal antibodies tested in this study were significantly less sensitive than the PV pool. However, the Argene pool was found to possess a significantly higher sensitivity than the Biotest pool, as already reported (2), whereas no significant difference was observed between the Argene and Chemicon or between the Chemicon and Biotest pools. We believe that from this point on, use of in vitro-generated pp65-positive PMN reference preparations will enable testing of new pools of monoclonal antibodies and evaluation of their actual test performance.

One of the major objectives of this study was to comparatively evaluate the optimal storage temperatures of fixed and unfixed slides. It was shown that unfixed slides are best stored at room temperature for up to 1 month, while it was already known that fixed slides are best stored at −80°C for unlimited periods. The optimal storage of unfixed slides at room temperature for a reasonable period would allow shipment at room temperature of cytospots to all over the world.

The newly developed procedure for in vitro preparation of cytospots finally allows us to address the problem of long-term storage of large batches of slides containing a predetermined number of pp65-positive PMN. In fact, thus far, only the sensitivity of the antigenemia assay for cytospin preparations from freshly collected blood samples has been compared to the sensitivity achieved for preparations from blood samples stored at room temperature or at 4°C for 24 to 72 h. In this respect, several reports, although unanimously recognizing a progressive loss in sensitivity of the antigenemia assay during

TABLE 5. Long-term effect of storage at room temperature of unfixed cytospots on HCMV antigenemia assay sensitivity with the PV pool

Duration of storage at room temperature	No. of replicates	Median no. of pp65-positive PMN (range)	*P* value[a]					Median no. (%) of pp65-positive PMN with degree of staining		
								Weak	Moderate	Strong
24 hours	6	69.5 (58–76)						15 (21)	48 (69)	6.5 (10)
10 days	6	69 (58–81)	NS[b]	NS				15 (22)	47 (68)	7 (10)
20 days	8	67 (59–73)	NS	NS	NS	0.0003	0.0055	15 (22)	45 (67)	7 (11)
30 days	5	70 (53–91)	NS	0.014				26 (37)	38 (54)	6 (9)
40 days	5	45 (43–47)	0.0015	0.0002				15 (34)	27 (60)	3 (7)
55 days	5	26 (20–28)	0.0005	0.0001				12 (46)	12 (46)	2 (8)
75 days	5	1 (0–5)	0.0001					1 (100)	0 (0)	0 (0)

[a] *t* test.
[b] NS, not significant.

storage, have provided somewhat controversial findings (1, 6, 7, 10).

In conclusion, we believe that standardization protocols and quality control programs may be developed for the HCMV antigenemia assay by taking advantage of the novel procedure for generating pp65-positive PMN in vitro.

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